IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Price et al.	§	**ART UNIT:**
	§	**1635**
FILED: June 14, 2001	§	
	§	**EXAMINER:**
SERIAL NO.: 09/881,635	§	**Angell, Jon E.**
	§	
FOR: Regulation Of the P21 Gene and Uses Thereof	§	
	§	**DOCKET:**
	§	**D6302**

Commissioner for Patents
P.O. BOX 1450
Alexandria, VA 22313

ATTENTION: Board of Patent Appeals and Interferences

TRANSMITTAL OF APPEAL BRIEF

Dear Sir:

Enclosed please find three (3) originals of the Appeal Brief for the above-referenced patent application.

The Commissioner is hereby authorized to charge Deposit Account No. 07-1185 in the total amount of $215 ($160 Appeal Fee + $55 Extension Fee) and any additional fee that may be required. Please return the enclosed postcard acknowledging receipt of this correspondence.

Respectfully submitted,

Date: August 22, 2003

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Mail Stop Appeal Brief - Patents
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APPELLANT'S BRIEF

This Brief is in furtherance of the Notice of Appeal filed in this case on June 16, 2003. The fees required under 37 C.F.R. §1.17(f) and any other required fees are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

In accordance with 37 C.F.R. §1.192(a), this Brief is submitted in triplicate.

INDEX OF SUBJECT MATTER

I. REAL PARTY IN INTEREST

The real party in interest is The University of Arkansas for Medical Sciences, the Assignee, as evidenced by an Assignment recorded in the Patent and Trademark Office at Reel 012650, Frame 0518 on February 20, 2002.

II. RELATED APPEALS AND INTERFERENCES

Appellant is aware of no other appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF THE CLAIMS

Originally claims 1-10 were filed with this Application. Claims 2, 4, 7, 9 and 10 were canceled by amendment. The pending claims 1, 3, 5, 6 and 8 are being appealed of which claims 1 and 6 are independent claims.

IV. STATUS OF AMENDMENTS

No claim amendment was made subsequent to the final rejection mailed January 14, 2003. Pending claims 1, 3, 5, 6 and 8 are shown in Appendix A.

V. SUMMARY OF THE INVENTION

Mice expressing a homozygous null mutation in *p21* were shown to be highly resistant to the deleterious effects of partial renal ablation. Removal of *p21* expression allowed the growth response in the kidney after partial ablation to be relatively more hyperplastic than in controls, so that the kidney work-load was then better accommodated by the increased growth (page 30, lines 1-3). The experimental results disclosed indicate that *p21* has a critical role in the functional and morphological consequences subsequent to the stress of renal ablation, including the development of glomerular sclerosis and hypertension, and that these symptoms are critically linked to the prominent role of *p21* in

regulating the cell cycle (page 29, lines 4-15). The striking effects seen in the *in vivo* mouse model experiments suggest that reducing or eliminating *p21* gene expression will be able to ameliorate or prevent the effects of acute renal stress or chronic renal failure (page 30, lines 9-14).

VI. ISSUES

35 U.S.C. §112

Whether claims 1, 3, 5, 6 and 8 are enabled under 35 U.S.C. §112, first paragraph.

VII. GROUPING OF CLAIMS

The rejected claims do not stand or fall together. Applicant considers that claims 1, 3 and 5 are separately patentable from claims 6 and 8. Applicant submits that the method of treating or preventing a pathophysiological state of a kidney in an individual (claim 1) is patentably distinct from the method of treating or

preventing chronic progressive renal failure in an individual by reducing or eliminating the expression of the *p21* gene in one or both kidneys of said individual (claim 6).

VIII. ARGUMENTS

Claims 1, 3, 5, 6 and 8 are rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. This rejection is respectfully traversed.

Claim 1 is drawn to a method for treating or preventing a pathophysiological state of a kidney in an individual, wherein the state is characterized by an undesirable level of cyclin-dependent kinase inhibitor activity in the kidney, comprising the step of reducing or eliminating the expression of the *p21* gene in the kidney of the individual.

Claim 6 is drawn to a method for treating or preventing chronic progressive renal failure in an individual, comprising the step of reducing or eliminating the expression of the *p21* gene in one or both kidneys of the individual, wherein the reducing or eliminating the expression of the *p21* gene results in the manipulation of cyclin-dependent kinase inhibitor activity in one or both kidneys.

The Examiner states that the claimed methods for treating a pathological state of the kidney in an individual by reducing or eliminating the expression of the *p21* gene are not supported by the specification so as to enable one skilled in the art to make and/or use the invention. The relevant issues are 1) that kidney disorders involve many different mechanisms, so that reducing or eliminating *p21* may not treat every kidney disorder; 2) the *p21* knock-out mouse is not a proper model for the claimed therapy; and 3) methods to efficiently reduce or eliminate *p21* expression in a kidney are unpredictable. These issues amount to a requirement of undue experimentation in order to perform the claimed methods. Applicant respectfully traverses the Examiner's rejections.

Applicant respectfully argues that the claims recite methods to treat or prevent pathophysiological states of kidney characteristic of chronic renal failure, which the specification shows to be alleviated in mice lacking functional *p21*. The specification discloses experiments employing partial renal ablation as a model of chronic renal failure from diverse causes (page 10, lines 9-11). Mice expressing a homozygous null mutation in *p21* are clearly demonstrated to be highly resistant to the deleterious effects of partial renal ablation. Removal of *p21* expression allowed the growth response in the kidney after partial ablation to be relatively more hyperplastic than in controls, so that the kidney work-load was then better accommodated by the increased growth (page 30, lines 1-3). Acute short-term kidney stress is known to provoke *p21* expression (page 3, lines 7-9), indicating that *p21* is responding according to its critical role in nuclear responses to environmental stress. Renal ablation causes a hyperplastic and hypertrophic reaction in the presence of *p21* expression, but the reaction in the absence of *p21* is more hyperplastic: this is indicated by the more than five-fold increase in PCNA expression in the renal epithelium of the remnant kidney in *p21* knock-out mice versus controls (Example

15 and Figure 6). These results indicate that the observed results are due to the inhibition of *p21* in its role as inhibitor of the cell-cycle, and that *p21* regulates the balance between hyperplasia and hypertrophy following renal ablation (page 11, lines 1-8).

The experimental results disclosed indicate that *p21* has a critical role in the functional and morphological consequences subsequent to the stress of renal ablation, including the development of glomerular sclerosis and hypertension, and that these symptoms are critically linked to the prominent role of *p21* in regulating the cell cycle (page 29, lines 4-15). The prior art to date has not shown such a causal link between the early adaptations to loss of kidney function and the progressive nature of renal disease (page 2, lines 11-20 and page 3, lines 1-5). The present application provides such a link in clearly demonstrating that controlling *p21* expression can prevent progressive renal disease *in vivo*. The striking effects seen in the *in vivo* mouse model experiments disclosed allow a reasonable prediction that reducing or eliminating *p21* gene expression will be able to ameliorate or prevent the effects of acute renal stress or chronic renal failure. The known methods in the art may therefore be used by skilled practitioners to practice

the present claims in targeting *p21* expression, thereby improving upon the current methods of treating or preventing long-term renal failure.

The specification discloses gene therapy, antisense therapy, and genetic manipulation as acceptable methodologies to accomplish a reduction or elimination of *p21* gene expression (page 9, lines 8-10). The Examiner contends that therapeutic methods known in the art are of an unpredictable nature, and the specification does not specifically identify any drugs, antisense molecules, or gene therapy methods that can be used to reduce or eliminate *p21* expression in the kidney of an animal; hence, an undue amount of experimentation would be required to practice such methods to efficiently reduce or eliminate the expression of a gene in an animal. Applicant respectfully disagrees.

Applicant submits that given the degree of experimentation that is routine in the art of regulation of gene expression, the amount of experimentation required of one skilled in the art to practice the claimed methods is not undue. The eight-factor *In re Wands* test for undue experimentation requires that a

conclusion of nonenablement be based on the evidence as a whole (M.P.E.P. §2164.01(a)). Although the existence of working examples is a factor, prophetic examples based on predicted results can be enabling if they disclose the claimed invention in such a way that one skilled in the art can practice the invention without undue experimentation (M.P.E.P §2164.02). Two other factors include the state of the prior art and the level of predictability in the art. Predictability refers to the ability of a skilled artisan to extrapolate the disclosed or known results to the claimed invention (M.P.E.P. §2164.03), and the fact that the experimentation required may be complex does not necessarily make it undue, if the art typically engages in such experimentation (M.P.E.P. §2164.01). The evidence supporting the ability of a skilled artisan to make or use the claimed invention using the application as a guide need not be conclusive, but merely convincing to the artisan; what one skilled in the art knew at the time of filing of the application is relevant (M.P.E.P. §2164.05).

Applicant respectfully submits that the disclosures of the present specification, coupled with the knowledge of one skilled in the art at the time of filing, enables one so skilled to practice the

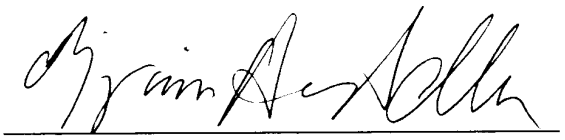
claimed invention without undue experimentation. According to the Examiner, the state of the art at the time of filing considers the success of gene and antisense therapy to be unpredictable, as stated in Paper No. 7 mailed August 15, 2002. Applicant submits, however, there was ongoing research and development of gene therapy and antisense methods at the time of filing of the present application. Crystal (*Science* 270:404-410 (1995)) states that "enough information has been gained from clinical trials to allow the conclusion that human gene transfer is feasible, can evoke biologic responses that are relevant to human disease, ... Adverse events have been uncommon and have been related to the gene delivery strategies, not to the genetic material being transferred" (see abstract). "Taken together, the evidence is overwhelming, with successful human gene transfer having been demonstrated in 28 ex vivo and 10 in vivo studies" (Crystal, page 405, third column, second paragraph; Tables 1 and 2). Branch (*TIBS* 23:45-50 (1998)) also acknowledges advances in the field of antisense therapy: "Today's peak specificity, whatever it is, will almost certainly rise as current strategies are optimized and advances in nucleic acid chemistry bring derivatives with fewer side effects" (page 47, second column, end of first full paragraph). Thus, in view of the

state of the art, Applicant respectfully submits that the level of development and skill in the art is sufficient to allow the practice of the present claims without undue experimentation, and accordingly respectfully requests that the rejection of claims 1, 3, 5, 6 and 8 under 35 U.S.C. 112, first paragraph, be withdrawn.

For the reasons given above, Applicants respectfully request that the decision of the Examiner should be reversed, and that claims 1, 3, 5, 6 and 8 be allowed.

Respectfully submitted,

Date: August 21, 2003



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CLAIMS ON APPEAL

1. A method for treating or preventing a pathophysiological state of a kidney in an individual, wherein said state is characterized by an undesirable level of cyclin-dependent kinase inhibitor activity in said kidney, comprising the step of reducing or eliminating the expression of the *p21* gene in said kidney of said individual.

3. The method of claim 1, wherein said pathophysiological state is selected form the group consisting of renal fibrosis, glomerulosclerosis, reduced filtration rates, and hypertension.

5. The method of claim 1, wherein the reduction or elimination of the expression of the *p21* gene is performed by a technique selected from the group consisting of drug therapy, and genetic manipulation.

6. A method for treating or preventing chronic progressive renal failure in an individual, comprising the step of reducing or eliminating the expression of the *p21* gene in one or both kidneys of said individual, wherein said reducing or eliminating the expression of the *p21* gene results in the manipulation of cyclin-dependent kinase inhibitor activity in one or both kidneys.

8. The method of claim 6, wherein the reduction or elimination of the expression of the *p21* gene is performed by a technique selected from the group consisting of drug therapy and genetic manipulation.

the folding of the protease sequences when added as separate molecules, both *in vitro* and *in vivo*[15]. One way in which cells change the quantitative properties of proteins is to make allosteric effectors; this method is reversible and requires the continual presence of the effector. Perhaps another method useful in say, terminal differentiation, is the production of separate steric chaperones that irreversibly change the properties of certain specific proteins by influencing their folding.

Acknowledgements

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A good antisense molecule is hard to find

Andrea D. Branch

Antisense molecules and ribozymes capture the imagination with their promise of rational drug design and exquisite specificity. However, they are far more difficult to produce than was originally anticipated, and their ability to eliminate the function of a single gene has never been proven. Furthermore, a wide variety of unexpected non-antisense effects have come to light. Although some of these side effects will almost certainly have clinical value, they make it hard to produce drugs that act primarily through true antisense mechanisms and complicate the use of antisense compounds as research reagents. To minimize unwanted non-antisense effects, investigators are searching for antisense compounds and ribozymes whose target sites are particularly vulnerable to attack. This is a challenging quest.

ANTISENSE STRATEGIES LOOK almost too easy on paper. Simple and elegant schemes can be drawn for both antisense oligodeoxynucleotides (ODNs – short DNA molecules intended to bind to and inhibit target RNAs through complementary Watson–Crick base pairing) and bioengineered ribozymes (catalytic RNA molecules intended to bind and cleave target RNAs). Scientists seek to use these molecules to ablate selected genes and thereby understand their functions, and pharmaceutical developers are working to find nucleic-acid-based therapies. However, the antisense field has been turned on its head by the discovery of 'non-antisense' effects, which occur when a nucleic acid drug acts on some molecule other than its intended target – often through an entirely unexpected mechanism. Non-antisense effects are not necessarily bad. Indeed, some may prove to be a boon to the pharmaceutical industry because they offer an added source of potency. However, their unpredictability confounds research applications of nucleic acid reagents.

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Non-antisense effects are not the only impediments to rational antisense drug design. The internal structures of target RNAs and their associations with cellular proteins create physical barriers, which render most potential binding sites inaccessible to antisense molecules. For Watson–Crick base pairing to occur, nucleic acid drugs must be complementary to exposed regions in their target RNAs and must co-localize with them. When these requirements are met, true antisense effects are enhanced, and unwanted non-antisense effects are minimized. However, optimization is a time-consuming process. Currently, effective nucleic acid drugs must be selected from large pools of candidates. Streamlined approaches for (irrational) *in vivo* selection are needed to speed the discovery of active molecules.

Non-antisense effects: quicksand for some, diamond mines for others

The potential of nucleic acid drugs to deliver 'exquisite specificity'[1] is frequently cited: antisense methods are credited with offering 'the specificity of the genetic code and the versatility of targeting any number of proteins'[2]; and it is said that a therapeutic ribozyme 'can be designed to interact only with its target, and the target is expected to appear only once in the genome, giving one a high degree of assurance that the target – and only that target – has been inhibited'[3]. However, it has never been proven that antisense drugs have the capacity to knock out just one gene, although both ODNs and bioengineered ribozymes can undeniably hit their intended targets[4,5]. The powerful appeal of antisense strategies has been a mixed blessing. The twin concepts that effective antisense reagents are easy to

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design and that they selectively home in on their targets have overshadowed the cautionary messages in articles such as 'Antisense has growing pains'[6], 'Can hammerhead ribozymes be efficient tools to inactivate gene function?'[7], and 'Does antisense exist? (It may, but only under very special circumstances)'[8].

The purpose of this article is to review the factors that make and break specificity in antisense applications and to discuss the need to judge therapeutic compounds and research reagents by separate standards. Only antisense molecules[9–11] and ribozymes[12,13] designed to inhibit RNA targets are considered here, but many of the principles apply to other nucleic acid drugs, such as those used to correct DNA mutations[14], to alter RNA splicing[15], and to control gene expression by forming triple helices with DNA (Ref. 16).

Non-antisense effects pose a dilemma for the pharmaceutical industry[17]. These effects include the stimulation of B-cell proliferation[18] and the inhibition of viral entry into cells[19], responses which are potentially useful. Non-antisense ODNs are already being developed as adjuvants to boost the efficacy of immunotherapies and vaccines[20]. Phase III clinical trials of ISIS 2922 (Ref. 21), a phosphorothioate oligonucleotide (S-ODN) that induces both antisense and non-antisense effects, are also under way in patients with cytomegalovirus-associated retinitis[22]. It is hoped that this compound's diverse mechanisms of action will yield a single drug that provides many of the benefits of combination therapy. However, as Anderson and colleagues have observed, characteristics that are advantages in pharmaceutical drugs can be disadvantages in research reagents[21]. Thus, a safe and effective nucleic acid drug that slows the progression of AIDS would be of tremendous value, even if it were to act by inhibiting a perplexing combination of viral proteins rather than by binding to HIV RNA as originally intended. However, this same compound would be useless as an agent to selectively destroy HIV RNA, and could be ruinous if used in experiments of HIV molecular biology without knowledge of its mechanism of action. Because a single, well-understood mechanism of action cannot be assumed, non-antisense effects create major difficulties for gene hunters. Years of investigation can be required to figure out what an 'antisense' molecule is actually doing, as discussed further below.

Non-antisense effects also have a downside for pharmaceutical developers. Because knowledge of their underlying mechanisms is typically lacking, non-antisense effects muddy the waters. They make true antisense drugs more difficult to design and harder to commercialize. Furthermore, they can be a source of toxicity.

All drugs are dirty: clinical benefit is the pharmaceutical gold standard

Stanley Crooke (Isis Pharmaceuticals) stresses that 'a vast body of experience says that no drug is entirely selective'[23]. Because biologically active compounds generally have a variety of effects, dose–response curves are always needed to establish a compound's primary pharmacological identity. Antisense compounds are no exception. As is true of all pharmaceuticals, the value of a potential antisense drug can only be judged after its intended clinical use is known, and quantitative information about its dose–response curves and therapeutic index is available.

It may be surprising to hear antisense molecules described in the same terms as conventional drugs, but, in fact, nucleic acid drugs should not be thought of as magic bullets. Their therapeutic use will require vigilant monitoring. Compared to the dose–response curves of conventional drugs, which typically span two to three orders of magnitude, those of antisense drugs extend only across a narrow concentration range. Both *in vitro* and *in vivo*, less than a factor of ten often separates the concentration producing no antisense effect from that producing the full antisense effect[22]. Steep dose–response curves commonly indicate that a drug has multiple, synergistic mechanisms of action[24]. A drug with a narrow therapeutic window can be potent and extremely valuable, but can also be tricky to use safely. Since the ratio of antisense to non-antisense effects drops sharply outside a restricted concentration range, it will be challenging to obtain consistent therapeutic results.

Mother Nature's cruel antisense jokes lead to tougher experimental standards

Their powerful allure and favorable press have often caused the problems associated with antisense reagents to be trivialized. In some cases, relaxed standards have been applied. Arthur Krieg (University of Iowa) provided insight into the need for stricter quality control when he shared the results of an informal poll. He reported that 'the estimate that many people have given me of the percentage of accurate published antisense papers ranges from 50% of them being accurate to 5% being accurate'[22].

As discussed previously, when an antisense molecule causes a biological effect, it can be extremely difficult to determine whether the change occurred because the reagent interacted specifically with its target RNA, or because some non-antisense reaction – involving other nucleic acids or proteins – was set in motion[8,25]. When attempting to distinguish between antisense and non-antisense effects, a common strategy has been to use an oligonucleotide in which the sequence of the antisense oligonucleotide is altered. Unfortunately, not all non-antisense effects can be readily detected by this approach, as illustrated by studies of antisense therapies for chronic myeloid leukemia. In this disease, a chromosomal translocation often produces the Philadelphia chromosome, resulting in the synthesis of an oncogenic fusion protein, BCR/ABL. The mRNA for this protein has been regarded as an ideal target for antisense therapies. Several groups have reported inhibition of leukemic cell proliferation by anti-BCR/ABL antisense oligonucleotides. In fact, Vaerman and co-workers cite 16 publications reporting promising findings[26]. However, they discovered that a disappointing, non-antisense mechanism was responsible for their own results, adding weight to studies showing that S-ODNs block proliferation through non-antisense mechanisms (reviewed in Ref. 26). Recent work indicates that cytotoxic ODN breakdown products are responsible for the antiproliferative effects observed[27]. These studies strongly underscore the need to test numerous control ODNs when carrying out antisense research, and to maintain a high index of suspicion.

C. A. Stein (Columbia University) has reviewed many 'non-sequence-specific' (non-antisense) effects caused by S-ODNs, providing dramatic examples of the havoc that has resulted when S-ODNs have unleashed their surfeit of cryptic information. S-ODNs are used because their modified backbones confer nuclease resistance. However, they bind avidly to many proteins, forming complexes with dissociation constants one to three orders of magnitude lower than those of phosphodiester ODNs. In a test of B-cell proliferation and differentiation, S-ODNs were two logs more potent than phosphodiester ODNs of the same sequence[28]. According to Stein, S-ODNs have 'bamboozled' many researchers by inducing biological effects that mimic, and are mistaken for, true and desired antisense effects[8,19].

Addressing the manifest need for stricter experimental standards, Arthur Krieg and C. A. Stein (editors of the journal *Antisense and Nucleic Acid Drug Development*) have published guidelines for designing antisense studies[1]. Recently, the need to use pure oligonucleotide reagents has been stressed. The selective publication of expected (positive) results is being actively discouraged. The confusion that has thus far occurred indicates that each new 'antisense' molecule needs to be tested exhaustively.

How close do current antisense techniques come to single-gene accuracy?

While the ability to knock out a single gene is a luxury in a pharmaceutical compound, specificity is a key feature of a reagent to be used in a research setting. Although single-gene accuracy is not essential for an experimental reagent to be useful, the extraneous perturbations it causes need to be identified. Additionally, as alternative approaches for selective gene ablation (such as the production of genetic knockouts) improve and become easier to carry out, it will be important to know how antisense techniques compare in terms of time, expense and selectivity. This comparison awaits additional information about antisense specificity.

Unfortunately, quantitative data about the magnitude of antisense-induced side reactions are limited. Most of the information is extrapolated from experiments in which the impact of an antisense compound is measured on only a small number of molecules: the intended target RNA, a housekeeping gene, and perhaps a few control RNAs. An antisense molecule is typically taken to be 'specific' if two criteria are met: (1) there is no gross loss of cell viability, and (2) the levels of the target RNA and its associated protein fall much more than those of the control RNAs. However, this type of experimental design is too limited in scope to provide information about global changes in the RNA and protein populations. It does not provide even a rough measure of the signal-to-total noise ratio. Unlike the analysis of Scatchard plots, which allows the interactions between a ligand and a complex mixture of proteins to be explored, this design looks at three or four RNAs and projects the impact on the remaining 10^5 genes. As an additional shortcoming, it provides no direct information about interactions between the antisense molecule and proteins, even though these interactions may lead to the major effects caused by 'antisense' molecules. Because it could provide a before-and-after snap-shot of the protein population, high-resolution two-dimensional gel electrophoresis[29] might shed light on the spectrum of changes induced by antisense molecules. However, a recent round-table discussion suggested that there are no published studies in which this technique has been utilized to evaluate antisense specificity[22].

So far, the concept that an antisense molecule can selectively knock out a single gene appears to have been untested. In the future, several techniques, in addition to two-dimensional gel electrophoresis, might be employed to investigate antisense specificity. For example, as the repository of sequenced genes grows, it will be possible to identify RNAs that contain regions complementary to an antisense molecule and to measure the impact of antisense treatments on these bystander molecules. In addition broad surveys of mRNA populations could be conducted. To identify changes induced by antisense treatments, RNA from treated and control cells could be reverse-transcribed and the resulting cDNA populations analyzed either by differential display, which separates cDNAs electrophoretically, or by hybridization to gene chips, which are being developed to allow the quantitative monitoring of gene expression patterns[30]. Should unanticipated changes be detected by such surveys, other techniques could be used to distinguish those caused by lack of specificity from those reflecting downstream consequences of the intended antisense reaction. Information about the number of accidental hits and about the nature of the interactions responsible for the changes in the expression of other genes would be useful and would guide future drug development. Today's peak specificity, whatever it is, will almost certainly rise as current strategies are optimized and advances in nucleic acid chemistry bring derivatives with fewer side effects. New compounds are currently under investigation[17,31] and additional derivatives can be expected in the future.

Theoretical limits of specificity

Theoretical calculations provide a useful perspective on antisense specificity. The haploid human genome contains about 3×10^9 bases. In a *random* sequence of this size, any sequence that is 17 nucleotides long or longer would have a high probability of occurring only once - of being unique. To knock out a single gene, an intervention would have to distinguish a 17-base perfect match from one with a single-base mismatch.

In considering whether ODNs have the requisite power of discrimination, it is crucial to know their mechanism(s) of action. These mechanisms may differ from cell type to cell type and may depend upon the exact nature of the target RNA and the ODN. However, there is strong evidence that in several systems, including *Xenopus* oocytes[32] and permeabilized cells[33], the target RNA is destroyed by the action of RNase H. RNase H activities cleave the RNA component of DNA–RNA hybrids. They do not require long hybrid regions as substrates. In fact, *in vitro*, RNase H can cleave a hybrid containing only 4 bp (Ref. 34). In *Xenopus* oocytes, as few as 10 bp are sufficient[35]. For standard ODNs, it is likely that 10 bp are also sufficient in human cells; in the case of certain chemically modified nucleotides, it is proven that as few as 7 bp can lead to cleavage[36]. Random sequences the length of the human genome contain an average of 3000 copies of each 10-nucleotide sequence (10-mer). Thus, it is extremely likely that any particular 10-mer will occur in many RNAs. When an ODN complementary to this 10-mer is introduced into a cell, all of the RNAs containing this 10-mer are at risk for RNase H-mediated cleavage. Of course, not all 3000 copies will be susceptible to cleavage: many will not be present in transcripts, and many that are present in transcripts will be inaccessible. However, if even 1% of the 3000 are hit, 30 genes will be directly affected. Furthermore, the number of 'at risk' sites is probably more than an order of magnitude greater than 3000 for two reasons: (1) ODNs typically contain 20 or more bases, each 20-mer contains 11 10-mers, and each 10-mer would be present 3000 times, on average; and (2) in all likelihood, RNase H does not require 10 consecutive bp for cleavage. Because RNase H requires only a short hybrid region, it is not possible to increase specificity by increasing the length of the ODN. In fact, increasing the length beyond the minimum is likely to have the opposite effect, by stabilizing binding to mismatched sequences, as illustrated in Fig. 1.

Based on studies performed in *Xenopus* oocytes, Wooll and co-workers concluded that it is probably not possible to obtain cleavage of an intended target RNA without also causing at least partial destruction of many non-targeted RNAs (Ref. 35). The ratio of intended to unintended hits will depend on a complex and unpredictable combination of factors that determine whether the antisense molecule and the potential targets co-localize and

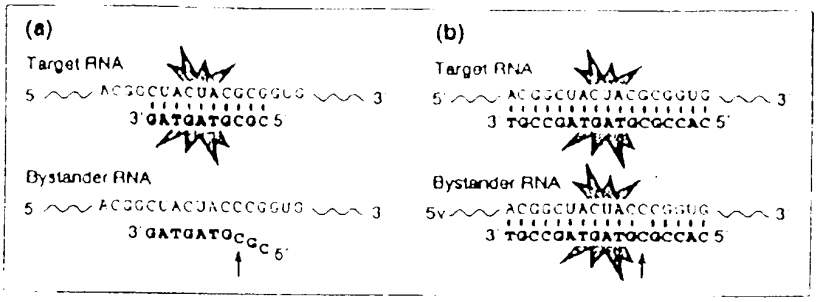

Figure 1

More is not always better. **(a)** A relatively short antisense ODN causes destruction of its intended target RNA but not a bystander RNA. This discrimination is possible because the ODN does not form enough base pairs with the bystander RNA to promote stable binding and RNase H-mediated cleavage. **(b)** A longer ODN annihilates both the target and the bystander, indiscriminately. From the standpoint of the gene hunter, an unfortunate situation exists. In general, an ODN short enough to discriminate between an RNA containing a perfect match and an RNA containing a one base mismatch is so short that its perfect complement occurs in many different RNAs in a human cell. Thus, although it can distinguish between perfect and imperfect matches, the ODN cannot selectively destroy its target RNA. To overcome this problem, the second generation of ODNs will need special design features to enhance their specificity. In the diagrams, the 'explosion' denotes RNA cleavage by RNase H. ODNs are presented in boldface type, and sequences complementary to all or part of the ODN appear in regular lettering with the remainders of the target and bystander RNAs depicted by wavy lines; black arrows identify a nucleotide mismatch between the bystander RNA and the ODN (the bystander and the target RNA differ at this position).

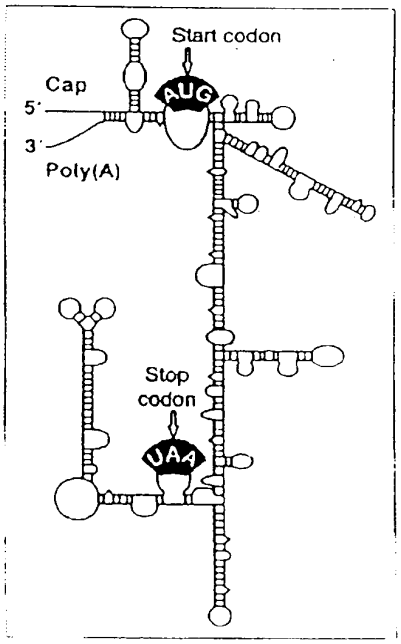

Figure 2

As illustrated by this secondary structure map of mouse β-globin mRNA, RNA molecules have an intricate array of intramolecular Watson–Crick bonds, which greatly diminish the portion of the molecule available for binding to antisense compounds and ribozymes. The positions of base pairs were determined by treating globin mRNA with structure-sensitive nucleases *in vitro*. Redrawn from Ref. 40, with kind permission.

whether the complementary sites in the RNAs are buried under proteins or are involved in intramolecular bonds that make them inaccessible. In the future, even as improvements in antisense chemistry reduce oligonucleotide binding to proteins, the specificity limits imposed by RNase H will remain and will be important to keep in mind when evaluating antisense strategies.

Target site recognition by bioengineered ribozymes is determined by Watson–Crick base pairing and thus has limits of specificity similar to those of ODNs. Ribozymes bind to their target RNAs through a recognition sequence of variable length. Somewhat counterintuitively, a ribozyme with the potential to form a larger number of base pairs with its target RNA does not necessarily have a greater power to discriminate between its intended target and a related bystander RNA than a ribozyme with a shorter recognition sequence. In fact, extending the length of the recognition sequence may reduce a ribozyme's ability to discriminate[37]. It remains to be determined whether there are recognition sequence lengths that are both short enough to allow RNAs that differ from the target at a single nucleotide to be spared cleavage and long enough to allow a unique RNA to be selectively destroyed[38]. It will not be surprising if bioengineered ribozymes are incapable of knocking out single genes, as contemplated by Bertrand and co-workers[7]. Most of these molecules are derived from either hammerhead or hairpin ribozymes[13]. In their natural setting, these ribozymes are covalently attached to their cleavage sites. They self-cleave precursor molecules of subviral (viroid) pathogens[39]. To fulfill their duties, these ribozymes have only to select their target site from the limited number of choices available in the same (small) RNA molecule. Thus, in terms of specificity, bioengineered ribozymes are expected to outperform their natural counterparts. Of course, besides binding to unintended RNAs through Watson–Crick and/or non-Watson–Crick interactions, ribozymes, like other RNAs, are highly charged molecules and have the potential to bind to cellular proteins, thereby producing biologically significant (non-antisense) effects.

As regards the theoretical limits of antisense specificity, it is important to remember that the genome is not a 'random sequence'. Sequences that constitute 'good' antisense targets in one RNA may occur in other RNAs at a higher or lower frequency than random chance would predict. One anecdote reveals how the redundancy of biological sequences could plague antisense methods. A conserved 350-base region at the 5′ end of the hepatitis C virus is considered to be a potential target for antisense drugs. This short region contains a particular 10-mer that is also present in 62 known human mRNAs (Ref. 25), and it contains two 17-mers that occur in known human DNA sequences. Ultimately, the tendency for biological sequences to be reused may limit the specificity of strategies that rely solely on Watson–Crick base pairing for recognition. This tendency will become amenable to detailed analysis soon, as more complete data about human gene sequences become available.

The three As of antisense-mediated gene ablation: access, access and access

Inside cells, it is obviously not possible to improve specificity by raising the temperature or changing the ionic strength, manipulations that are commonly used to reduce background binding in nucleic acid hybridization experiments *in vitro*. Thus, alternative strategies are needed to enhance specificity within cells. One approach has been to deploy multiple antisense compounds, each directed against a different site in the same target RNA and thereby achieve annihilation by molecular triangulation. In addition, successful efforts have been made to exploit the fact that not all

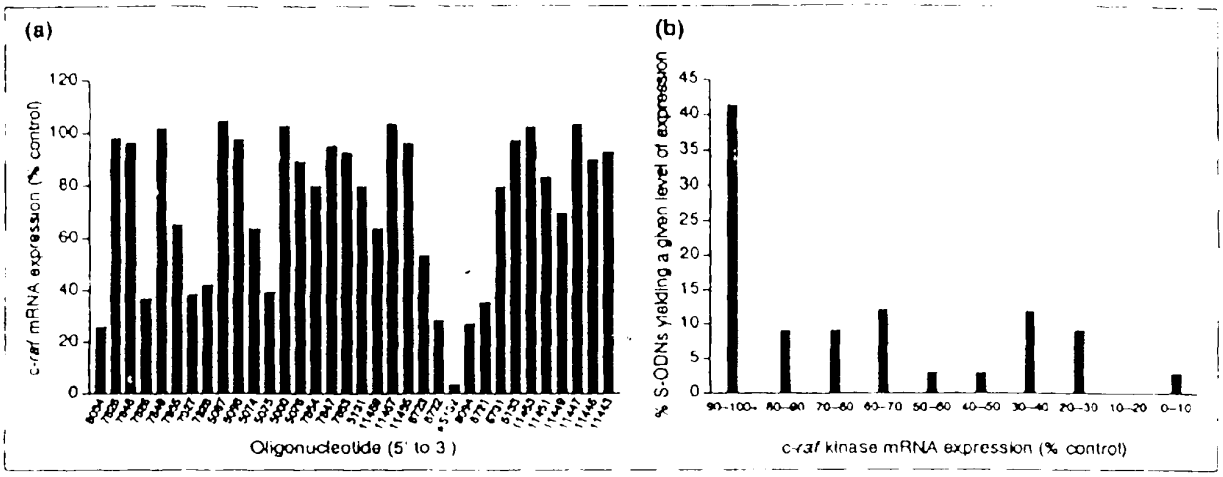

Figure 3

Superior S-ODNs can be found, but they are in the distinct minority. **(a)** Northern hybridization analysis revealed that, of 34 S-ODNs applied to A549 lung carcinoma cells, only one (5132, marked by an asterisk) caused a greater than fivefold reduction in the level of the target, c-*raf* kinase mRNA. Redrawn from Ref. 42, with kind permission. **(b)** Treatment with the majority of the S-ODNs had minimal effect and resulted in levels of the target mRNA that were 50% or more of the level in control cells.

portions of an RNA molecule are equally exposed. If a 10-mer complementary to an antisense ODN occurs in an accessible site in a target RNA and in a protected portion of a bystander, the target will be preferentially destroyed. The challenge is to identify antisense molecules that are complementary to vulnerable sites in target RNAs. This is hard to do. RNAs are complex molecules with intricate internal structures[40], as illustrated by the diagram of β-globin mRNA (Fig. 2).

Recent studies emphasize the extent to which native RNA structure restricts the binding of ODNs. Milner and co-workers[41] tested the ability of 1938 ODNs (ranging in length from monomers to 17-mers) to bind to a 122-nucleotide RNA representing the 5' end of β-globin mRNA. They found that 'surprisingly few' ODNs bound stably to the mRNA, and concluded that binding is probably 'confined to those regions in the RNA which provide an accessible substructure'[41]. Using short (7 and 8 nucleotides) antisense molecules modified with C-5 propyne pyrimidine and phosphorothioate internucleotide linkages, Wagner and co-workers[36] also determined that the structure of the target RNA is a 'major determinant of specificity'.

Because it is very difficult to predict what portions of an RNA molecule will be accessible *in vivo*, effective antisense molecules must be found empirically by screening a large number of candidates for their ability to act inside cells. Monia and co-workers used northern hybridization to screen 34 20-nt long S-ODNs complementary to c-*raf* kinase and found only one that yielded a greater than fivefold reduction in the target mRNA (Fig. 3a; Ref. 42). Thus, only 3% of the antisense molecules tested in this system were highly effective (Fig. 3b), 40% had almost no effect[42].

Like those of ODNs, ribozyme target sites also vary in their accessibility. Chen and co-workers[43] directly demonstrated that cellular proteins and ribonucleoprotein complexes, such as ribosomes, can prevent ribozyme-mediated cleavage. They showed that a reporter gene was ribozyme-insensitive in wild-type *Escherichia coli* but was ribozyme-sensitive in a 'slow ribosome' mutant. In an accompanying editorial, John Burke (University of Vermont) remarked, 'The simple picture of ribozymes diffusing to, binding, and then cleaving an unstructured RNA is hopelessly oversimplistic'[44].

Rational and irrational design strategies are converging

At any one moment, a combination of the inherent structure of the RNA and its collection of bound proteins limits the number of accessible sites on RNA molecules, thereby providing a basis for specificity. Binding is the rare exception rather than the rule, and antisense molecules are excluded from most complementary sites (see Fig. 4). Since accessibility cannot be predicted, rational design of antisense molecules is not possible. Because design rules are lacking, effective antisense molecules are typically selected from 20–50 candidates in a time-consuming and expensive process that promises to become even more elaborate. If tests of 50 molecules identify good candidates, tests of thousands of compounds should identify better ones. If thousands are to be tested, how should they be designed? Should their sequences be based solely on their potential to form a linear series of Watson–Crick base pairs with the target, or should nucleation sites be included, as they are in naturally occurring antisense RNAs (Ref. 45)? What about non-canonical base-pair interactions, and structural features such stem loops?

The relationship between accessibility to ODN binding *in vitro* and vulnerability to ODN-mediated antisense inhibition *in vivo* is beginning to be explored, and will continue to be an active area of research in the future. It is not yet clear whether *in vitro* screening techniques of the sort used by Milner and co-workers[41] will identify ODNs that are effective *in vivo*. With so many possible sequences to choose from, and the likelihood that *in vitro* studies will not always predict *in vivo* efficacy, straightforward new screening techniques need to be developed for use in cells.

Conclusions

The original concept that ODNs and ribozymes are exquisitely specific and easy to design has been jolted by the discovery of numerous mechanisms of action, leading to non-antisense effects, and the finding that most Watson–Crick binding sites in intended target RNAs

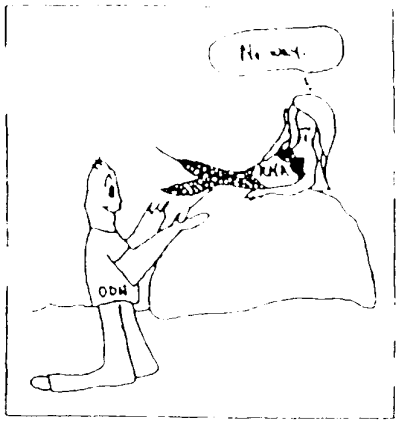

Figure 4
The structure of most potential target sites makes them inaccessible to antisense molecules and ribozymes.

are inaccessible. The time and expense necessary to screen large numbers of potential antisense molecules and ribozymes, and to carefully monitor their *in vivo* effects, raise the stakes for those seeking to use them as genetic probes. Although questions of their ultimate specificity remain, there is growing evidence that antisense molecules can be useful pharmacological tools when applied carefully[17]. In addition, certain non-antisense effects promise to be valuable therapeutically and will be fascinating to investigate. Because non-antisense effects are not currently predictable, rules for rational design cannot be applied to the production of non-antisense drugs. These effects must be explored on a case-by-case basis.

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Transfer of Genes to Humans: Early Lessons and Obstacles to Success

Ronald G. Crystal

Enough information has been gained from clinical trials to allow the conclusion that human gene transfer is feasible, can evoke biologic responses that are relevant to human disease, and can provide important insights into human biology. Adverse events have been uncommon and have been related to the gene delivery strategies, not to the genetic material being transferred. Human gene transfer still faces significant hurdles before it becomes an established therapeutic strategy. However, its accomplishments to date are impressive, and the logic of the potential usefulness of this clinical paradigm continues to be compelling.

Human gene transfer is a clinical strategy in which the genetic repertoire of somatic cells is modified for therapeutic purposes or to help gain understanding of human biology (*1*, *2*). Essentially, gene transfer involves the delivery, to target cells, of an expression cassette made up of one or more genes and the sequences controlling their expression. This can be carried out ex vivo in a procedure in which the cassette is transferred to cells in the laboratory and the modified cells are then administered to the recipient. Alternatively, human gene transfer can be done in vivo, in a procedure in which the expression cassette is transferred directly to cells within an individual. In both strategies, the transfer process is usually aided by a vector that helps deliver the cassette to the intracellular site where it can function appropriately (*1*, *2*).

Once considered a fantasy that would not become reality for generations, human gene transfer moved from feasibility and safety studies in animals to clinical applications more rapidly than expected by even its most ardent supporters (*1–3*). It is not the purpose of this review to detail all human protocols that have been proposed, but to use examples from the available information regarding ongoing human trials (*3*) to define the current status of the field.

How Is Human Gene Transfer Carried Out?

The choice of an ex vivo or in vivo strategy and of the vector used to carry the expression cassette is dictated by the clinical target. The vector systems for which data are available from clinical trials (retroviruses, adenoviruses, and plasmid-liposome complexes) transfer expression cassettes through different mechanisms and thus have distinct advantages and disadvantages for different applications (*1*, *2*).

Vectors. Replication-deficient, recombinant retrovirus vectors can accommodate up to 9 kb of exogenous information (Fig. 1). Retroviruses transfer their genetic information into the genome of the target cell, and thus, theoretically, the target cell's genotype is permanently modified (*1*, *2*, *5*). This is an advantage when treating hereditary and chronic disorders, but it has risks, including the potential for toxicity associated with chronic overexpression or insertional mutagenesis (for example, if the proviral DNA randomly disrupts a tumor suppressor gene or activates an oncogene). The use of retrovirus vectors is limited by the sensitivity of the vector to inactivation, by the fact that target cells must proliferate in order to integrate the proviral DNA into the genome, and by production problems associated with recombination, rearrangements, and low titers (*1*, *2*, *5*). Retrovirus vectors have been used almost entirely in ex vivo gene transfer trials.

Adenovirus vectors in current use accommodate expression cassettes up to 7.5 kb (*1*, *2*, *6*). These vectors enter the cell by means of two receptors: a specific receptor for the adenovirus fiber and $\alpha_v\beta_3$ (or $\alpha_v\beta_5$) surface integrins that serve as a receptor for the adenovirus penton (*7*) (Fig. 2). Adenovirus vectors are well suited for in vivo transfer applications because they can be produced in high titers (up to 10^{13} viral particles/ml) and they efficiently transfer genes to nonreplicating and replicating cells (*8*). The transferred genetic information remains epichromosomal, thus avoiding the risks of permanently altering the cellular genotype or of insertional mutagenesis. However, adenovirus vectors in current use evoke nonspecific inflammation and antivector cellular immunity (*9*). These responses, together with the epichromosomal position of the expression cassette, limit the duration of expression to periods ranging from weeks to months. Thus adenovirus vectors will have to be readministered periodically to maintain their persistent expression. Although it is unlikely that

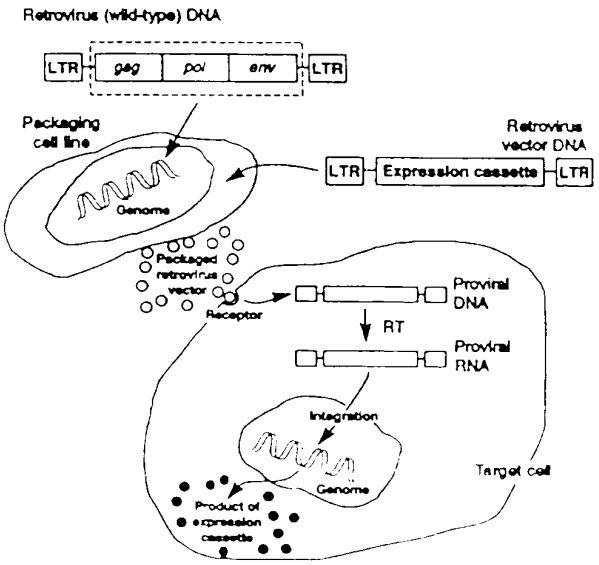

Fig. 1. Retrovirus vector design, production, and gene transfer. Retroviruses are RNA viruses that replicate through a DNA intermediate. The retrovirus vectors administered to humans all use the Maloney murine leukemia virus as the base. The *gag*, *pol*, and *env* sequences are deleted from the virus, rendering it replication-deficient. The expression cassette is inserted, and the infectious replication-deficient retrovirus is produced in a packaging cell line that contains the *gag*, *pol*, and *env* sequences that provide the proteins necessary to package the virus. The vector with its expression cassette enters the target cell via a specific receptor. In the cytoplasm, the reverse transcriptase (RT) carried by the vector converts the vector RNA into the proviral DNA that is randomly integrated into the target cell genome, where the expression cassette makes its product.

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repeat administration will be risky, it is not known whether antibodies directed against vector capsid proteins will limit the efficacy of repetitive administration of these vectors (9). Adenovirus vectors have been used only in in vivo human trials.

In theory, plasmid-liposome complexes have many advantages as gene transfer vectors, in that they can be used to transfer expression cassettes of essentially unlimited size, cannot replicate or recombine to form an infectious agent, and may evoke fewer inflammatory or immune responses because they lack proteins (10) (Fig. 3). The disadvantage of these vectors is that they are inefficient, requiring that thousands of plasmids be presented to the target cell in order to achieve successful gene transfer. The available data are not sufficient to determine if repetitive administration of liposomes or DNA poses safety risks. Plasmid-liposome complexes have been used only in in vivo human trials.

Expression cassettes and clinical targets. Human gene transfer studies fall into two categories: marking and therapeutic (Table 1). The marking studies use expression cassettes with bacterial antibiotic-resistant genes, which allow the genetically modified cells to be identified (Table 1). Because the marking genes have no function (other than to permit selection of the modified cells in vitro), the trials using marker genes have been designed to demonstrate the feasibility of human gene transfer, to uncover biologic principles relevant to human disease, and to evaluate safety. These trials have mostly used retrovirus vectors and have focused on malignant disorders or on human immunodeficiency virus (HIV) infection.

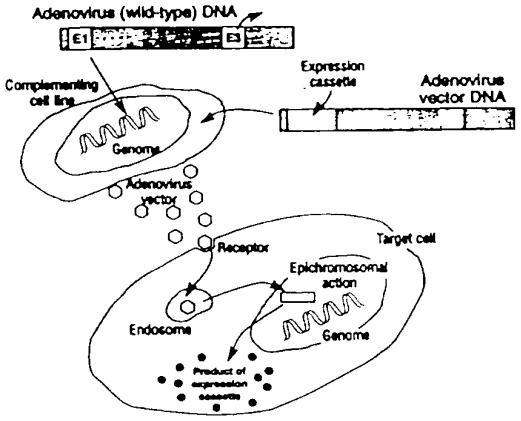

Fig. 2. Adenovirus vector design, production, and gene transfer. Adenoviruses are DNA viruses with a 36-kb genome. The wild-type adenovirus genome is divided into early (E1 to E4) and late (L1 to L5) genes. All adenovirus vectors administered to humans use adenovirus serotypes 2 or 5 as the base. The ability of the adenovirus genome to direct production of adenoviruses is dependent on sequences in E1. To produce an adenovirus vector, the E1 sequences (and E3 sequences if the space is needed) are deleted. The expression cassette is inserted, and the vector DNA is transfected into a complementing cell line with E1 sequences in its genome. The adenovirus vector with its expression cassette is E1$^-$ and thus incapable of replicating. The vector binds to the target cell through an interaction of the adenovirus fiber and penton, each to a specific receptor; moves into a cytoplasmic endosome; and breaks out and delivers its linear, double-stranded DNA genome with the expression cassette into the nucleus, where it functions in an epischromosomal fashion to direct the expression of its product.

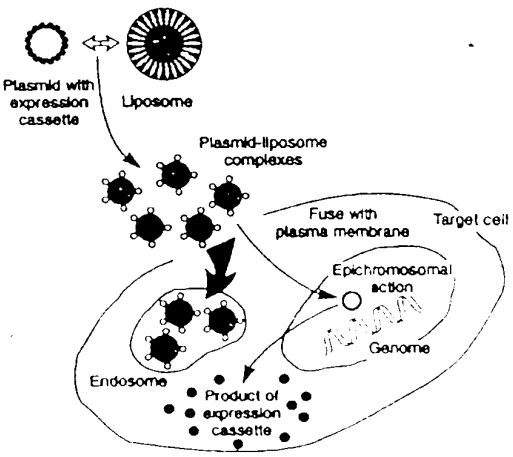

Fig. 3. Plasmid-liposome complex design and gene transfer. The liposomes used in human gene transfer trials have various compositions, but typically include synthetic cationic lipids. The positively charged liposome is complexed to the negatively charged plasmid with its expression cassette. The complexes enter the target cell by fusing with the plasma membrane. The vector does not have an inherent macromolecular structure that conveys information to enable efficient translocation of the plasmid to the nucleus. Consequently, most of the newly introduced genetic material is wasted as it is shunted to cytoplasmic organelles. When used in vivo, it is likely that most, if not all, of the plasmids that reach the nucleus function in an epischromosomal fashion.

The therapeutic trials seek to transfer expression cassettes carrying genes that will evoke biologic responses that are relevant to the treatment of human disease, and to demonstrate that this can be accomplished safely. The therapeutic studies have used retrovirus vectors, adenovirus vectors, or plasmid-liposome complexes. All of the therapeutic trials have been directed toward monogenic hereditary disorders or cancer.

What Has Really Been Accomplished?

Feasibility of gene transfer. Probably the most remarkable conclusion drawn from the human trials is that human gene transfer is indeed feasible. Although gene transfer has not been demonstrated in all recipients, most studies have shown that genes can be transferred to humans whether the strategy is ex vivo or in vivo, and that all vector types function as intended. Taken together, the evidence is overwhelming, with successful human gene transfer having been demonstrated in 28 ex vivo and 10 in vivo studies (Table 1).

In the ex vivo studies with retrovirus vectors, successful gene transfer to humans has been shown by the transfer of marker genes to various classes of T cells (*11–16*), to stem cells in blood and marrow (*16–27*), to tumor-infiltrating lymphocytes (TILs) (*11*, *28*, *29*), to neoplastic cells of hematopoietic lineage (*16*, *17*, *20*, *21*, *25*, *26*), and to neoplastic cells derived from solid tumors (Table 1). Although there is variation among ex vivo clinical trials in the proportion of genetically marked cells recovered from the recipients, retroviral vector DNA or marker gene–derived mRNA or both have been observed in cells collected after periods ranging from several weeks to 36 months after administration.

Retrovirus vectors also have been used to transfer therapeutic genes ex vivo, with success demonstrated by the fact that the modified cells exhibit their altered phenotype in vivo for up to 36 months (Table 1). Typically, the expression cassette containing the therapeutic gene also contains an antibiotic-resistance gene, permitting the ex vivo selection of genetically modified cells recovered from the recipient. Successful gene transfer has been demonstrated in cells recovered from children with adenosine deaminase (ADA) deficiency after transfer of the normal ADA complementary DNA (cDNA) to autologous T cells, cord blood, and placental cells (*30–32*); from individuals with solid tumors after transfer of cytokine cDNAs in autologous vaccine strategies to fibroblasts, TILs, or tumor cells (*33–37*); from individuals with familial hypercholesterolemia after transfer of the low-density lipoprotein (LDL) receptor cDNA to autologous hepatocytes (*38*, *39*);

from HIV$^+$ siblings after transfer of a chimeric T cell receptor cDNA to blood T cells of a twin (*40*), and from individuals with tumors who received autologous marrow transplants after transfer of the multidrug resistance 1 cDNA to autologous blood CD34$^+$ stem cells (*41*). A retrovirus vector has also been used in vivo to successfully transfer a p53 antisense cDNA to lung carcinoma cells (*42*). Finally, in a combined ex vivo–in vivo strategy for treatment of brain neoplasms, gene transfer to tumor cells has been observed after xenogeneic cells (murine fibroblasts whose genome had been modified with amphotropic packaging sequences) infected with a retrovirus vector containing an expression cassette with the herpes simplex thymidine kinase (HSTK) gene were introduced into the tumor (*43*).

In in vivo studies with adenovirus vectors, several studies have shown that direct administration of a vector containing the normal human cystic fibrosis transmembrane conductance regulator (CFTR) cDNA to the nasal or bronchial epithelium of individuals with cystic fibrosis (CF) results in transfer of the CFTR cDNA-containing expression cassette to the epithelium, where CFTR mRNA or protein is expressed for at least 9 days (*44–50*) (Table 1). Direct administration of a plasmid-liposome complex containing an expression cassette with the CFTR cDNA to the nasal epithelium of individuals with CF resulted in expression of CFTR mRNA in the epithelium (*51*). Finally, plasmid-liposome complexes have

Table 1. Summary of studies showing that transfer of genes to humans is feasible. Data shown are based on published articles and abstracts and on RAC-mandated biannual reports of principal investigators as of the RAC meeting of 8 to 9 June 1995. Abbreviations used for vector study type are RV, retrovirus; Ad, adenovirus; PL, plasmid-liposome complex; M, marker-type study; and T, therapeutic-type study. Abbreviations used for gene products are Neo^R, neomycin phosphotransferase; Hygro, hygromycin phosphotransferase; HSTK, herpes simplex thymidine kinase; ADA, adenosine deaminase; LDLR, low-density lipoprotein receptor; TNF, tumor necrosis factor α; CD4 zeta-R, chimeric T cell receptor; MDR-1, multidrug resistance 1; IL-4, interleukin 4; GM-CSF, granulocyte macrophage colony-stimulating factor; CFTR, cystic fibrosis transmembrane conductance regulator; and B7 + β_2, histocompatibility locus antigen class I–B7 + β_2 microglobulin. Except for Neo^R, Hygro, and HSTK, all genes are cDNAs. Abbreviations used for target cells are TIL, tumor-infiltrating lymphocytes; EBV, Epstein-Barr virus; HIV, human immunodeficiency virus 1; and CTL, cytotoxic T lymphocytes. All target cells are autologous unless otherwise specified. Abbreviations used to characterize study populations are AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; ALL, acute lymphocytic leukemia; ca, carcinoma; and CF, cystic fibrosis. Under in vivo evidence of gene transfer, a plus sign indicates a report of transfer or expression (or both) of an exogenous gene in cells obtained from one or more individuals in the study; time listed is the longest time after administration that gene transfer or expression was observed.

Vector study type	Gene product	Target cells	Study population	In vivo evidence of gene transfer	Principal investigator	Reference number
RV-M	Neo^R	TIL	Melanoma	+ 2 months	Rosenberg, S. A.	(*28*)
RV-M	Neo^R	TIL	Melanoma	+ 3 months	Lotze, M. T.	(*29*)
RV-M	Neo^R	Marrow	AML	+36 months	Brenner, M. K.	(*16, 17*)
RV-M	Neo^R	Marrow	Neuroblastoma	+29 months	Brenner, M. K.	(*18*)
RV-M	Neo^R	Marrow	Neuroblastoma	+20 months	Brenner, M. K.	(*18*)
RV-M	Neo^R	Marrow	CML	+ 5 months	Deisseroth, A. B.	(*20*)
RV-M	Neo^R	Marrow	AML, ALL	+12 months	Cornetta, K.	(*21*)
RV-M	Neo^R	CD4$^+$, CD8$^+$, blood, TIL	Melanoma, renal cell ca	+	Economou, J. S.	(*11*)
RV-M	Neo^R	CD34$^+$ blood, marrow	Multiple myeloma	+18 months	Dunbar, C. E.	(*22, 23*)
RV-M	Neo^R	CD34$^+$ blood, marrow	Breast ca	+18 months	Dunbar, C. E.	(*23, 24*)
RV-M	Neo^R	Marrow	AML	+12 months	Brenner, M. K.	(*25*)
RV-M	Neo^R	Normal twin blood T cells†	Identical twins, 1 HIV$^+$	+ 4 months	Walker, R. E.	(*12*)
RV-M	Neo^R	Blood, marrow	CML	+	Deisseroth, A. B.	(*26*)
RV-M	Neo^R	CD34$^+$ blood	Metastatic ca, lymphoma	+15 days	Schuening, F. G.	(*27*)
RV-M	Neo^R	EBV-specific CTL‡	Ca, leukemia	+ 7 months	Heslop, H. E.	(*14, 15*)
RV-M	Hygro + HSTK	CD8$^+$ HIV *gag* specific, CTL§	HIV$^+$, lymphoma	+14 days	Greenberg, P.	(*13*)
RV-T	ADA	Blood T cells	ADA deficiency	+36 months	Blaese, R. M.	(*30, 31*)
RV-T	ADA	Cord blood cells	ADA deficiency	+18 months	Blaese, R. M.	(*30, 32*)
RV-T	LDLR	Hepatocytes	Familial hypercholesterolemia	+ 4 months	Wilson, J. M.	(*38, 39*)
RV-T	TNF	TIL	Melanoma	+	Rosenberg, S. A.	(*33*)
RV-T	IL-2	Tumor cells‖	Metastatic ca	+	Rosenberg, S. A.	(*36*)
RV-T	IL-2	Neuroblastoma‖	Metastatic ca	+	Brenner, M. K.	(*35*)
RV-T	CD4 zeta-R	Normal twin blood T cells†	Identical twins, 1 HIV$^+$	+ 4 months	Walker, R. E.	(*40*)
RV-T	MDR-1	Blood CD34$^+$	Breast ca	+	Deisseroth, A. B.	(*41*)
RV-T	IL-4	Fibroblasts¶	Metastatic ca	+	Lotze, M. T.	(*34*)
RV-T	GM-CSF	Melanoma‖	Melanoma	+	Dranoff, G.	(*37*)
RV-T	Anti-sense p53	Lung ca	Lung ca	+ 1 days	Roth, J. A.	(*42*)
RV-T*	HSTK	Tumor cells	Glioblastoma	+	Oldfield, E. H.	(*43*)
Ad-T	CFTR	Nasal, airway epithelium	CF	+ 9 days#	Crystal, R. G.	(*44, 45*)
Ad-T	CFTR	Nasal epithelium	CF	+	Welsh, M. J.	(*46, 47*)
Ad-T	CFTR	Nasal epithelium	CF	+	Welsh, M. J.	(*48*)
Ad-T	CFTR	Airway epithelium	CF	+ 5 days**	Wilson, J. M.	(*49*)
Ad-T	CFTR	Nasal epithelium	CF	+	Boucher, R. C.	(*50*)
PL-T	CFTR	Nasal epithelium	CF	+ 4 days	Goddes, D. M.	(*51*)
PL-T	B7 + β_2	Melanoma	Metastatic ca	+ 3 days	Nabel, G. J.	(*52*)
PL-T	B7 + β_2	Colorectal ca	Metastatic ca	+	Ruben, J. T.††	(*53, 54*)
PL-T	B7 + β_2	Renal cell ca	Metastatic ca	+	Vogelzang, N.††	(*54, 55*)
PL-T	B7 + β_2	Melanoma	Metastatic ca	+	Hersh, E.††	(*56*)

*This study used a mixed ex vivo–in vivo strategy, in which a xenogeneic fibroblast cell line was modified with a retrovirus to produce an amphotropic retrovirus vector containing an expression cassette with the genes for Neo^R + HSTK, and the modified retrovirus-producing cell line was administered directly into the tumor. †Blood T cells from a normal identical twin modified with an expression cassette and then administered to an HIV$^+$ twin. ‡Autogeneic. §The HSTK gene used as a marker gene. ‖Autologous tumor cells modified with an expression cassette, lethally irradiated, and then administered as a "vaccine." ¶Autologous fibroblasts modified with an expression cassette, lethally irradiated, and then administered together with autologous unmodified tumor cells as a "vaccine." #Messenger RNA at 9 days; vector DNA at 15 days. **A few + cells were observed at 90 days. ††Collaborative study, different institutions.

been used to transfer the human leukocyte antigen (HLA)–B7 and β2 microglobulin cDNAs directly to solid tumors in vivo, with consequent expression of the transfer cassette being seen in the tumor (52–56).

Relevant biologic responses. No human disease has been cured by human gene transfer, and it is not clear when this will be accomplished. However, several studies have demonstrated that therapeutic genes transferred to humans by means of retrovirus, adenovirus, and plasmid-liposome vectors can evoke biologic responses that are relevant to the gene product and to the specific disease state of the recipient (Table 2). Most of the studies demonstrating biologic efficacy have focused on monogenic hereditary disorders, where it is rational to believe that, if the normal gene product could be appropriately expressed at the relevant site, the abnormal biologic phenotype could be corrected.

Severe combined immunodeficiency–ADA deficiency is a fatal recessive disorder caused by mutations in the gene encoding ADA; these mutations cause accumulation of adenosine and 2′-deoxyadenosine, which are toxic to lymphocytes (57). Affected children are unable to generate normal immune responses and develop life-threatening infections. The normal ADA cDNA was transferred ex vivo with a retrovirus vector into T lymphocytes of two children with this disorder, and the modified T cells were expanded in the laboratory and periodically infused into the autologous recipients (30, 31). This resulted in an increase in T cell numbers and in the ADA levels in circulating T cells. The two children now have partially reconstituted immune function, as demonstrated by T cell cytokine release, cytotoxic T cell activity, isohemagglutinin production, and skin test responses to common antigens. In addition, three infants with ADA deficiency who received autologous infusions of cord blood $CD34^+$ stem cells modified ex vivo with a retrovirus vector containing the normal ADA cDNA have also shown evidence of increased numbers of blood T cells and increased ADA levels in T cells (30, 32). The results of the ADA studies are difficult to interpret, because none of these trials have been controlled and the recipients have also received the standard therapy of enzyme infusions with mono-methoxypolyethylene glycol-bovine ADA. Despite these caveats, these observations are consistent with the conclusion that this ex vivo gene transfer strategy evokes biologic responses that are relevant to treatment of ADA deficiency.

Familial hypercholesterolemia is a fatal disorder caused by a deficiency of LDL receptors in the liver that are secondary to mutations in the LDL receptor genes (38, 39, 58). The consequences are high levels of serum cholesterol and LDL cholesterol, premature atherosclerosis, and myocardial infarction. A retrovirus vector was used ex vivo to transfer the normal LDL receptor cDNA to autologous hepatocytes obtained by partial liver resection of an individual with this disorder (38, 39). After reinfusion of the modified hepatocytes into the liver via the portal vein, there was a reduction in LDL cholesterol and in the ratio of LDL to high-density lipoprotein over 18 months, which is consistent with the concept that the corrected cells functioned in vivo to internalize and metabolize LDL cholesterol appropriately. Like the ADA deficiency studies, this study was partially compromised because other therapies were being administered. Furthermore, the LDL receptor gene mutations were mild and could have responded to experimental variables other than the transferred gene (58). However, similar transfer of autologous hepatocytes modified ex vivo to other individuals with more severe mutations of the LDL receptor gene demonstrated partial correction of a variety of lipoprotein-related metabolic parameters, which is consistent with the conclusion that this gene transfer strategy did evoke a relevant response (38).

Cystic fibrosis is the most common lethal hereditary disorder in North America (59). It is caused by mutations in the CFTR gene, a gene coding for an adenosine 3′,5′-monophosphate (cAMP)–regulatable chloride channel in the apical epithelium. As a result of these mutations, the airway epithelium is deficient in CFTR function. This leads to chronic airway infection and inflammation and progressive respiratory derangement. There is compelling logic to the argument that these lung derangements could be prevented if CFTR function could be restored in these cells (60). It is difficult to assess CFTR function in the airway epithelium in vivo in humans, but the nasal

Table 2. Data from human gene transfer studies in which transfer of genetic material has evoked a biologic response that is relevant to the underlying disease.

Disease category	Disease	Strategy	Vector	Gene product*	Target cells	Relevant biologic response	Principal investigator	Reference number
Hereditary	ADA deficiency	Ex vivo	Retrovirus	ADA	Blood T cells and cord blood $CD34^+$ stem cells	Partial restoration of immune response	Blaese, R. M.	(30–32)
	Familial hypercholesterolemia	Ex vivo	Retrovirus	LDLR	Hepatocytes	Partial correction of lipid abnormalities	Wilson, J. M.	(38, 39)
	Cystic fibrosis	In vivo	Adenovirus	CFTR	Nasal epithelium	Partial correction of potential difference abnormalities across the nasal epithelium	Welsh, M. J. Crystal, R. G.	(46, 47) (44, 62)
	Cystic fibrosis	In vivo	Plasmid-liposome complex	CFTR	Nasal epithelium	Partial correction of potential difference abnormalities across the nasal epithelium	Geddes, D. M.	(51)
Acquired	Solid tumors	In vivo	Plasmid-liposome complex	HLA-B7 + β_2	Tumor cells†	Specific immune response to tumor	Nable, G. J. Rubin, J. Vogelzang, N. Hersh, E.	(52) (53, 54) (54, 55) (54, 56)
		Ex vivo	Retrovirus	IL-4	Fibroblasts‡§	Specific and nonspecific immune response to tumor	Lotze, M.	(34)
		Ex vivo	Retrovirus	IL-2	Neuroblastoma‡	Specific and nonspecific immune response to tumor	Brenner, M. K.	(35)

*ADA, adenosine deaminase deficiency; LDLR, low-density lipoprotein receptor; CFTR, cystic fibrosis transmembrane conductance regulator; HLA-B7 + β_2, histocompatibility locus antigen class I B7 + β_2 microglobulin; IL-4, interleukin-4. †Direct administration to melanoma, colorectal carcinoma, or renal cell carcinoma. ‡Lethally irradiated, used as a vaccine. §Combined with lethally irradiated, unmodified autologous tumor cells.

epithelium has been used as a surrogate to test the hypothesis that in vivo transfer of the normal CFTR cDNA will correct the functional consequences of CFTR deficiency (47, 62). The parameters measured relate to the observation that the deficiency in CFTR causes an abnormal potential difference between the nasal epithelial surface and subcutaneous tissues. Although the nasal epithelium is not identical to the airway epithelium, two of three studies with adenovirus vectors (44–47, 50, 62) and one with plasmid-liposome complexes (51) have demonstrated that in vivo transfer of the CFTR cDNA to the nasal epithelium evokes a partial correction of these potential difference abnormalities for 1 to 2 weeks.

There are also studies in which human gene transfer appears to have initiated biologic responses that are relevant to therapy for an acquired disorder. These are all "tumor vaccine" studies, based on the hypothesis that exaggerated local expression of an immune-related cytokine might help activate the immune system sufficiently to recognize tumor antigens and control the growth of tumor cells. In one ex vivo study, a retrovirus vector was used to transfer the interleukin-4 (IL-4) cDNA to autologous fibroblasts (34). The cells were then irradiated and implanted subcutaneously in the donor together with irradiated, unmodified, autologous tumor cells. In some recipients, this evoked infiltration with $CD3^+$ T cells and tumor-specific $CD4^+$ T cells at the immunization site, as well as enhanced expression of cell adhesion molecules on capillary endothelium. In another trial, autologous neuroblastoma cells modified ex vivo with a retrovirus to contain the IL-2 cDNA were lethally irradiated and implanted subcutaneously (35). In some individuals, this evoked systemic augmentation of $CD16^+$ natural killer cells and tumor-specific $CD8^+$ cytotoxic T cells and eosinophilia. Finally, in four trials, in vivo plasmid-liposome complexes were used to transfer a heterologous HLA class I–B7 cDNA and the β_2 microglobulin cDNA directly to solid tumors (52–56). In several patients, there was evidence that the gene transfer process initiated amplification of the numbers of detectable, circulating, tumor-specific cytotoxic T cells.

Insights into human biology. Experience with marking studies has shown that human gene transfer can yield important insights into human biology by making it possible to track the fate of genetically marked cells in a recipient. For example, when stored autologous marrow is used to rescue a patient from the suppression of marrow function that complicates high-dose chemotherapy for late-stage malignancy, the individual may subsequently develop a recurrence of the malignancy. Gene transfer marking studies have helped answer the question of whether the recurrence is secondary to a residual tumor in the patient or is derived from malignant cells contaminating the reinfused banked marrow. Several studies that used an ex vivo strategy with a retrovirus vector to mark marrow cells with a neomycin resistance (neo^R) gene and then reinfused the marked marrow have demonstrated that contamination of the autologous marrow with malignant cells is common (11, 16–25). These observations have led to more attention being focused on purging banked autologous marrow of contaminating neoplastic cells before they are reinfused.

There are a number of strategies being developed for the use of ex vivo gene transfer to protect autologous T cells from infection with the HIV-1. None will work, however, if autologous T cells manipulated in the laboratory and then reinfused into an HIV^+ individual have a short biologic half-life. The life-span of an autologous T cell in HIV^+ individuals has been evaluated in identical twin pairs in which one twin is HIV^+ and the other is HIV^- (12). A retrovirus vector was used ex vivo to transfer the neo^R gene into the T cells from the normal twin, and the genetically marked cells were then reinfused into the HIV^+ twin. Some $CD4^+$ and $CD8^+$ marked T cells (or their progeny) survived for at least 10 months, providing a baseline to allow future studies to compare the fate of T cells that have been genetically modified to prevent HIV infection.

In a strategy to prevent reactivation of Epstein-Barr virus (EBV) and the accompanying associated lymphoproliferative disease after bone marrow transplantation, allogenic EBV-specific cytotoxic T cells (CTL) were genetically marked with a retrovirus vector, and the cells were infused into individuals at risk (15, 16). This preliminary study suggested that EBV-specific allogenic cells may help control EBV-associated complications of marrow transplantation, and the use of the marker genes demonstrated that the infused EBV-specific CTL persisted in the recipients for 10 weeks.

Two types of therapeutic studies support the biologic concept that minimal correction of a genotype can have significant phenotypic consequences. In the ex vivo study of retrovirus-mediated transfer of the LDL receptor cDNA into autologous hepatocytes in patients with familial hypercholesterolemia, liver biopsy several months after reinfusion of the modified hepatocytes showed that at most 5% of the total hepatocyte population expressed the normal gene in vivo (38, 39, 62). Despite this minimal correction, in some of the recipients there were changes in LDL-related parameters that suggested LDL receptor function in the liver had been partially restored. Partial phenotypic correction has also been observed in most of the trials of adenovirus- and plasmid-liposome complex–mediated in vivo transfer of the CFTR cDNA to the nasal epithelium in CF, even though the amount of gene transfer and expression has been limited to a small fraction of the target cells (44–47, 50, 51, 62).

Finally, when adenovirus vectors are administered to experimental animals, the animals quickly develop circulating neutralizing antibodies directed against the vector (9). In two studies of administration of adenovirus vectors to the airways of individuals with CF, no circulating neutralizing antibodies were detected (44, 45, 49). This is an important observation, because the expression cassette delivered by adenovirus vectors remains epichromosomal, and thus the vector will have to be readministered as its expression wanes. Although it is possible that there are local antibodies to the vectors in these individuals (9), the lack of a systemic immune response to such an antigen load is encouraging in that it suggests that antibodies to vectors may not be a major factor limiting persistent vector expression in humans when the lung is repeatedly dosed (64).

Safety of gene transfer. The theoretical safety concerns regarding human gene transfer are not trivial. For the individual recipient, there is the possibility of vector-induced inflammation and immune responses, of complementation of replication-deficient vectors leading to overwhelming viral infection, and (for the retrovirus vectors) of insertional mutagenesis. There are also theoretical issues that are important to society, including concerns about modifying the human germ line and about protecting the environment from new infectious agents generated from gene transfer vectors carrying expression cassettes with powerful biologic functions.

There have been adverse events in the human gene transfer trials, including inflammation induced by airway administration of adenovirus vectors (44–50, 65) and by administration to the central nervous system of a xenogenic producer cell line releasing a retrovirus vector (43, 66). However, compared with the total numbers of individuals undergoing gene transfer, adverse events have been rare and have been related mostly to the dose and the manner in which the vectors were administered. Shedding of viral vectors in the in vivo trials was very uncommon and was limited in extent and time (42, 44–50, 65). No novel infectious agents generated from recombination of the transferred genome and the host genome or other genetic information have been detected, nor has any replication-competent virus related to the vector. Cells modified ex vivo with retrovirus vectors have been infused repeti-

tively without adverse effects (13, 30, 31, 35), adenovirus vectors have been administered repetitively in vivo to the nasal (48) and bronchial epithelium safely (64, 67), and plasmid-liposome complexes have been administered repetitively to tumors in vivo without complications (52–56). Finally, human gene transfer has not been implicated in initiating malignancy, although the numbers of recipients and time of observation will have to be much greater to allow definitive conclusions regarding this issue.

What Are the Obstacles to Successful Human Gene Transfer?

With the successes of the human gene transfer trials have come the sobering realities of the drug development process. Some of the problems are generic for the field, and some are specific for each vector.

Inconsistent results. All of the human gene transfer studies have been plagued by inconsistent results, the bases of which are unclear. For example, in the two children with ADA deficiency receiving intermittent infusions of autologous T cells modified ex vivo with the normal ADA cDNA, the resulting proportion of ADA^+ circulating T cells has varied from 0.1 to 60% (30, 31). In the CF trials, there is evidence that adenovirus vectors and plasmid-liposome complexes can transfer the normal CFTR cDNA to the respiratory epithelium, but expression is observed in at most 5% of the target cells and is not seen in all recipients (44–51, 65). Further, an appropriate biologic response to gene transfer (correction of the abnormal potential difference across the nasal epithelium) has been observed in some patients in most, but not all, of the studies of CFTR cDNA transfer (44–47, 50, 51, 62). In most of the ex vivo marrow-marking trials, successful gene transfer is observed intermittently (Table 1).

Humans are not simply large mice. There have been several surprise examples, in which predictions from gene transfer studies in experimental animals have not been borne out in human safety and efficacy trials. In tumor vaccine studies intended to evoke a tumor-directed immune response, there is no convincing evidence (other than anecdotal case reports) that tumors regress, despite the promising observations in experimental animals (34, 37, 52–56). It has also become apparent that studies in experimental animals may not necessarily predict the toxicology of vectors in humans. In one patient with CF in whom 2×10^9 plaque-forming units of an adenovirus vector containing the CFTR cDNA were administered to the lung, a transient local and systemic inflammatory syndrome was evoked, despite the fact that no clinically apparent toxicity was observed in rodents and nonhuman primates receiving 1000-fold greater doses by the same route (45). Likewise, in an ex vivo–in vivo strategy to treat glioblastoma, transfer of xenogenic retrovirus-producing cells to the tumor was accomplished without significant adverse effects in experimental animals, but the human studies have been associated with central nervous system toxicity related to transfer of the cell line to the tumor (43, 66).

Production problems. There are significant hurdles in vector production that must be overcome before large clinical trials can be initiated. Generation of replication-competent virus is observed in production of clinical-grade retrovirus and adenovirus vectors; and lack of reproducibility, aggregation, and contamination with endotoxin complicate the production of clinical-grade plasmid-liposome complexes (68).

The perfect vector. The ideal gene transfer vector would be capable of efficiently delivering an expression cassette carrying one or more genes of the size needed for the clinical application. The vector would be specific for its target, not recognized by the immune system, stable and easy to reproducibly produce, and could be purified in large quantities at high concentrations. It would not induce inflammation and would be safe for the recipient and the environment. Finally, it would express the gene (or genes) it carries for as long as required in an appropriately regulated fashion (69).

This ideal vector is conceptually impractical, because the human applications of gene transfer are broad, and the ideal vector will likely be different for each application. Clinical experience to date suggests that retrovirus, adenovirus, and plasmid-liposome vectors all need refinement, but each is relatively well suited for the clinical targets at which they have been directed. Further, the technology is now available to create designer vectors that can be optimized for each application. Among the design hurdles for all vectors are the need to increase the efficiency of gene transfer, to increase target specificity and to enable the transferred gene to be regulated. Reproducible production of large amounts of pure vector is a hurdle for all classes of vectors. Some of the vector-specific hurdles are reduction of the risk for insertional mutagenesis in retrovirus vectors, minimization of the amount of immunity and inflammation evoked by the adenovirus vectors, and enhancement of the translocation of the gene to the nucleus for the plasmid-liposome complexes.

There is considerable interest in developing new vectors, but there is controversy as to which vector class is most likely to succeed, particularly for use in in vivo applications. There are two philosophical camps in vector design: viral and nonviral. The viral proponents believe that the most efficient means to deliver an expression cassette in vivo is to package it in a replication-deficient recombinant virus. The logic supporting this approach is the knowledge that viruses are masterful at reproducing themselves, and thus have evolved strategies to efficiently express their genetic information in the cells they infect. The nonviral proponents concede this argument but believe that the redundant anti-immune and inflammatory host defenses against viruses may be a risk to recipients, will limit the duration of expression as the infected cells are recognized by the immune system, and may hinder the efficacy of repeat administration of the vectors. Thus, nonviral vector aficionados believe it is rational to start from scratch to design safe, efficient, gene transfer strategies. In contrast, the viral camp believes that it is best to start with something that works but then to circumvent the replication, immune, and inflammation risks inherent in their use by appropriate vector design. It is most likely that these philosophical differences will eventually disappear as new classes of vectors are designed that incorporate features of viral and nonviral vectors, as dictated by specific clinical applications.

Future Prospects

None of the drug development problems facing human gene transfer are insurmountable, but each will take time to solve. However, the logic underlying the potential usefulness of human gene transfer is compelling; and put in a context in which the human genome project will provide 80,000 to 100,000 human genes that could be used in expression cassettes for human gene transfer, the potential impact of this technology for innovative therapies and increased understanding of human biology is enormous.

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4. In the United States, human gene transfer trials are regulated at the local level (by institutional review boards and biosafety committees) and at the national level [by the National Institutes of Health's Recombinant DNA Advisory Committee (RAC) and the Food and Drug Administration (FDA)]. The RAC mandates that principal investigators report twice yearly regarding the status of all approved protocols. The RAC reports and protocols are available from the Office of Recombinant DNA Activities, National In-

stitutes of Health, Suite 323, 6006 Executive Boulevard, MSC 7052, Bethesda, MD [illegible], USA. Human data in this review are derived from published articles and abstracts and from the December 1994 and June 1995 RAC investigator reports. Because the RAC reports are mandated, frequently updated, and public, they are an accurate gauge of the status of the field, although they are not peer reviewed. Since the first human trial was begun in 1989, there has been an explosion of interest in human gene transfer. In the United States alone, more than 100 human gene transfer protocols have been approved by the RAC, and 697 individuals have participated in human gene transfer trials under RAC-approved protocols (summary data, *RAC Report*, June 1995).
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The Nematode *Caenorhabditis elegans* and Its Genome

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Over the past two decades, the small soil nematode *Caenorhabditis elegans* has become established as a major model system for the study of a great variety of problems in biology and medicine. One of its most significant advantages is its simplicity, both in anatomy and in genomic organization. The entire haploid genetic content amounts to 100 million base pairs of DNA, about 1/30 the size of the human value. As a result, *C. elegans* has also provided a pilot system for the construction of physical maps of larger animal and plant genomes, and subsequently for the complete sequencing of those genomes. By mid-1995, approximately one-fifth of the complete DNA sequence of this animal had been determined. *Caenorhabditis elegans* provides a test bed not only for the development and application of mapping and sequencing technologies, but also for the interpretation and use of complete sequence information. This article reviews the progress so far toward a realizable goal—the total description of the genome of a simple animal.

Caenorhabditis elegans has many attractive features as an experimental system (*1*). The life cycle is simple and rapid, with a 3-day generation time, and populations can be grown with ease on agar plates or in liquid, usually by using *Escherichia coli* as a food source. These populations normally consist of only self-fertilizing hermaphrodites, but cross-fertilization is also possible, with the male sexual form. The option of reproduction by either selfing or crossing leads to very convenient genetics so that mutants can readily be generated, propagated, and analyzed (*2*). A simple freezing protocol permits stable storage of all strains, which retain viability indefinitely in the frozen state.

The animal, about 1 mm long when fully grown, is completely transparent at all stages of development. Both development and anatomy are essentially invariant among wild-type individuals. At maturity, all adult hermaphrodites contain 959 somatic nuclei and fewer than 2000 germ cell nuclei. Despite its low cell number, *C. elegans* has fully differentiated tissues corresponding to those of more complicated animals. The transparency and rapid development allow direct examination of cell division and differentiation in living animals with Nomarski microscopy. The small size of the animal also permits reconstruction of the entire anatomy at the ultrastructural level with serial section electron microscopy. However, the

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